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Amelioration of murine autoimmune pancreatitis by targeted LT R inhibition and anti-CD20 treatment

Wanner-Seleznik, Gitta M ; Reding, Theresia ; Chen, Rong ; Gupta, Anurag Kumar ; Lenggenhager, Daniela ; Browning, Jeffrey ; Segerer, Stephan ; Heikenwalder, Mathias ; Graf, Rolf

Abstract: Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis, for which treatment options, especially the long-term management, are limited. The only therapy that has been established and accepted so far is corticosteroids, but the relapse rate is significant. In the current study, we discern the effector mechanisms of targeted LT R pathway inhibition using LT R-Ig. Furthermore, the efficacy of LT R-Ig therapy is compared with the depletion of immune cell subsets (CD4+ and CD20+), which are suggested to play a pathological role in AIP development. Three well-established mouse models of AIP were used to examine treatment efficacies and mechanisms. Tg(Ela1-Lta,b) mice represent a genetic model, in which AIP develops spontaneously. In MRL/Mp and IL-10^{-/-} mice, AIP is induced by repeated polyinosinic:polycytidylic acid injection. Mice with AIP were treated with anti-CD20, anti-CD4 mAbs, or targeted LT R-Ig. LT R-Ig and anti-CD20 treatment led to significant improvement of AIP, including a decrease in autoantibody production and pancreatic inflammation in Tg(Ela1-Lta,b) and IL-10^{-/-} mice. The molecular mechanism of this beneficial effect possibly involves the downregulation of Stat3 and noncanonical NF- κ B activation. Anti-CD4 treatment reduced Th1 and Th2 signature but did not alleviate AIP. Additionally, in contrast to anti-CD20 or anti-CD4 treatments, blocking LT R signaling disrupted tertiary lymphoid organs in all three models. We demonstrate that treatment with LT R-Ig or anti-CD20 Ab alleviated murine AIP. LT R-Ig treatment for AIP was effective in both lymphotoxin-dependent and lymphotoxin-independent AIP models, possibly because of its dual anti-inflammatory and antiautoimmune mechanisms.

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Amelioration of Murine Autoimmune Pancreatitis by Targeted LT β R Inhibition and Anti-CD20 Treatment

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ABSTRACT

Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis, for which treatment options, especially the long-term management, are limited. The only therapy that has been established and accepted so far is corticosteroids, but the relapse rate is significant. In the current study, we discern the effector mechanisms of targeted LT β R pathway inhibition using LT β R-Ig. Furthermore, the efficacy of LT β R-Ig therapy is compared with the depletion of immune cell subsets (CD4⁺ and CD20⁺), which are suggested to play a pathological role in AIP development. Three well-established mouse models of AIP were used to examine treatment efficacies and mechanisms. Tg(Elal-Lta,b) mice represent a genetic model, in which AIP develops spontaneously. In MRL/Mp and IL-10^{-/-} mice, AIP is induced by repeated polyinosinic:polycytidylic acid injection. Mice with AIP were treated with anti-CD20, anti-CD4 mAbs, or targeted LT β R-Ig. LT β R-Ig and anti-CD20 treatment led to significant improvement of AIP, including a decrease in autoantibody production and pancreatic inflammation in Tg(Elal-Lta,b) and IL-10^{-/-} mice. The molecular mechanism of this beneficial effect possibly involves the downregulation of Stat3 and noncanonical NF- κ B activation. Anti-CD4 treatment reduced Th1 and Th2 signature but did not alleviate AIP. Additionally, in contrast to anti-CD20 or anti-CD4 treatments, blocking LT β R signaling disrupted tertiary lymphoid organs in all three models. We demonstrate that treatment with LT β R-Ig or anti-CD20 Ab alleviated murine AIP. LT β R-Ig treatment for AIP was effective in both lymphotoxin-dependent and lymphotoxin-independent AIP models, possibly because of its dual anti-inflammatory and antiautoimmune mechanisms. *ImmunoHorizons*, 2020, 4: 688–700.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare disease, but its prevalence is rising. This might be due to improved clinical assessment but also due to an overall increase in autoimmune diseases in industrialized countries. Consequently, the interest in treating

patients effectively with novel therapies will also increase in the future. Thus, thorough understanding of the cellular and molecular mechanisms driving AIP is urgently needed to develop novel therapies supporting current standard of care. With improved understanding of AIP and its distinct clinical profiles and variable association with a systemic IgG4 disease process (1),

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Abbreviations used in this article: aCD4, anti-CD4; aCD20, anti-CD20; ADM, acinar-to-ductal metaplasia; AIP, autoimmune pancreatitis; IM, immunomodulatory; LF, lactoferrin; LT, lymphotoxin; pDC, plasmacytoid dendritic cell; Poly IC, polyinosinic:polycytidylic acid; TF, transcription factor; TLO, tertiary lymphoid organ; Treg, regulatory T cell.

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AIP has been classified into type 1 and type 2 AIP: In type 1 AIP, the pancreas is affected as part of a systemic IgG4-positive disease and is characterized by lymphoplasmacytic infiltration accompanied by high endothelial venules and germinal center formation, hypergammaglobulinemia (IgG4), and vasculitis (2). Type 2 AIP is a fibroinflammatory duct-centric type with granulocyte epithelial lesions and pancreatic duct destruction without IgG4-positive cells or systemic involvement. Between the two entities, type 1 AIP is the more common form worldwide.

The cause of AIP is not completely understood. As suggested by its name, the immune system, both cellular and humoral components, is involved in the pathophysiology of AIP. However, it is still debated whether the immune system induces or only maintains AIP (3). One mechanism that possibly contributes to AIP development is a local inflammatory reaction. The release of proinflammatory cytokines may attract self-reactive T cells to initiate damage to local target tissues. In this context, autoimmunity does not initiate disease but may contribute to its chronicity (4). Based on the observation that lymphotoxin (LT) α and β are involved in the local inflammatory reaction during AIP, a transgenic mouse model with pancreas-specific LT expression was generated (5). This transgenic mouse model [*Tg(Elal-Lta,b)*] recapitulates several features of human AIP. Type 1 AIP in *Tg* mice is characterized by 1) the development of Abs against pancreatic self-antigens, 2) the presence of anti-nuclear Ab, 3) the rise in total serum IgG, and 4) extrapancreatic manifestation, mainly in the kidney in the form of IgG deposits in the glomeruli. In *Tg(ELal-Lta,b)* mice, the localized inflammation in the pancreas developed into AIP and systemic autoimmune disease between 9 and 12 mo of age, suggesting that local inflammatory reactions are involved in AIP pathogenesis. The *Tg(ELal-Lta,b)* model is an aetiologically relevant preclinical animal model, with spontaneous disease initiation; therefore, it is an ideal model for studying disease pathogenesis and for discovering new treatment strategies for AIP.

Currently, AIP patients are treated with corticosteroids, which promptly reduce inflammation and lead to morphological and clinical remission in most cases. Despite the high initial response in the case of type 1 AIP, the disease is predicted to relapse in 30–50% of patients. On the contrary, in type 2 AIP, disease relapses are rather uncommon (6). Although there is a general agreement that steroids are the ideal initial treatment, there is no clear consensus regarding the treatment of disease relapses (7). An additional high dose of steroids is the most commonly used treatment for managing disease relapse in type 1 AIP (7). In some instances of refractory or recurrent AIP, a steroid-sparing maintenance with immunomodulatory (IM) drugs is used. Experimental studies in mice have indicated that T cells have a major role in AIP induction and that cyclosporine and rapamycin could be alternatives to standard steroid treatment (8). Although approximately half of the patients with recurrent AIP might benefit from IM treatment, a significant group is resistant to IMs or intolerant to steroids. For such patients, treatment directed against B cells (e.g., with the anti-CD20 [aCD20] Ab rituximab) was shown to be effective. A few single case reports (9, 10) and a recent study at the Mayo Clinic (11) yielded

encouraging results regarding the use of rituximab in the setting AIP. Based on a limited follow-up, the risks of disease relapse and treatment-related side effects were low. Although these observational data showed a definite benefit of rituximab for treating IgG4-related diseases, the molecular mechanisms of this therapy are still unclear. Therefore, further studies to investigate the biological effects of rituximab and refining new therapeutic strategies are needed.

In the *Tg(Elal-Lta,b)* model, we have previously shown that in contrast to corticosteroids, which only diminished inflammation, inhibition of LT β receptor signaling (LT β R-Ig) was able to abrogate autoimmunity. To explore the benefits of LT β R-Ig treatment, in this study, we aimed at investigating mechanisms behind the effectiveness of LT β R inhibition. Furthermore, we tested LT β R-Ig treatment in other preclinical AIP models and compared its efficacy over other treatment approaches such as the depletion of B cells using aCD20 treatment. As cellular immune responses also contribute to the pathology of AIP, we included an arm of anti-CD4 (aCD4) treatment, leading to the depletion of CD4⁺ T cells and regulatory T cells (Tregs).

MATERIALS AND METHODS

Animal husbandry and samples

Animals were maintained under specific pathogen-free conditions, and experiments were approved and conform to the guidelines of the Swiss Animal Protection Law, Veterinary Office (Zurich, Switzerland). MRL/Mp mice were purchased from The Jackson Laboratory. Both male and female *Tg(Elal-LTab)* mice were used for the described experiments; however in MRL/Mp and IL-10^{−/−} animals, AIP is only inducible in female animals (12, 13).

RNA extraction

RNA was extracted and used for real-time PCR as described previously (14, 15) or with a newly established method using the Precellys 24 dual homogenizer with MagNA Lyser Green Beads from Roche Applied Science. In short, a small piece of snap-frozen tissue was transferred to a tube containing beads, 650 μ l of lysis buffer (QIAGEN) was immediately added, and the tube was immediately transferred to the Precellys and homogenized once at 6000 rpm for 30 s. The RNA was extracted following the QIAGEN RNeasy Mini Kit extraction protocol with an on-column DNase digestion step. Purified RNA was reversely transcribed into cDNA using qScript cDNA SuperMix von Quantabio, according to the manufacturer's protocol.

Real-time PCR

For mRNA expression analysis, real-time PCR was performed using specific TaqMan probes (Applied Biosystems). Real-time PCR was run on a 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) under standard conditions. Transcript levels were quantified using 18S RNA (Applied Biosystems) as a reference and normalized.

Histology and immunohistochemistry

Paraffin (3- μ m) sections of pancreas were stained with H&E or various primary and secondary Abs. Paraformaldehyde (4%) fixed

and paraffin-embedded tissue was incubated in Ventana buffer, and staining was performed on a NEXES immunohistochemistry robot (Ventana Medical Systems) using an iVIEW DAB Detection Kit (Ventana Medical Systems) or on a BOND-MAX (Leica Biosystems) system. Deparaffinization and antigen retrieval was performed in a PT Link pretreatment module with target retrieval solutions and stained on an Autostainer Link 48 (Dako, Glostrup, Denmark). Abs as summarized below.

The Abs used are as follows: B220 (clone RA3-6B; host: rat [550286; BD Pharmingen]; dilution 1:50); CD3 (host: rabbit [IR503; Dako]; ready to use); F4/80 (clone BM8; host: rat [BMA T2006; BMA Biomedicals]; dilution 1:100); Ki67 (clone SP6; host: rabbit [Ab 16667; Abcam]; dilution 1:200); Rel A (host: rabbit [RB-1638-P0; LabVision]; dilution 1:500); Rel B (host: rabbit [Sc-226; Santa Cruz Biotechnology]; dilution: 1:400); pStat3 (host: rabbit; dilution 1:100); PNad (host: rat [sc19602; Santa Cruz Biotechnology]; dilution 1:250); FDCM-1 (host: rat [51320; BD Pharmingen]; dilution 1:60); CD21/35 (host: rabbit [55553817; BD Pharmingen]; dilution 1:2000); CD4 (host: rat; dilution 1:50); and CD8 (host: rat; dilution 1:200).

Immunohistochemistry of kidneys was performed on paraffin-embedded materials as previously described (16). In brief, the sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide to block endogenous peroxidases. Endogenous biotin was blocked by the Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). An autoclave oven (or microwave treatment) was used for heat-based Ag retrieval. Incubation with the primary Ab was performed for 1 h or overnight. Incubation with biotinylated secondary Abs (Vector Laboratories) was followed by the avidin-biotin complex reagent (Vector Laboratories). 3'-3'-Diaminobenzidine (Sigma, Taufkirchen, Germany) with metal enhancement was used as a detection system. Consecutive sections were stained for mouse IgG (Vector Laboratories). Glomerular staining was quantified for IgG deposits in the mesangium and in peripheral glomerular capillaries using semiquantitative scores (from 0 to 3).

Paraffin-embedded pancreas specimens were immunostained for amylase and CK19. Acinar-to-ductal metaplasia (ADM) lesions were manually counted in a blinded fashion across the entire pancreas slide of individual mice. ADM was identified according to 1) the loss of amylase content, 2) positivity for CK19, and 3) structural reorganization into tubular complexes.

The primary Abs used for immunofluorescence staining were rabbit anti- α -amylase, fractionated antiserum (1:2000, A8273; Sigma), and rat anti-mouse cytokeratin 19 (1:25; DSHB; TROMA-III, developed by R. Kemler, Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany) followed by the incubation with matched secondary Abs Alexa Fluor 594 goat anti-rabbit IgG (1:200, A11012; Thermo Fisher Scientific) and Alexa Fluor 488 goat anti-rat IgG, respectively (1:200, A1106; Thermo Fisher Scientific). Nuclei were visualized by DAPI staining.

Samples were analyzed by researchers and pathologist in a blinded fashion without previous knowledge of the experimental groups.

Treatment with polyinosinic:polycytidylic acid

Polyinosinic:polycytidylic acid (Poly IC) was purchased by Sigma (P1530) and applied in 6-wk-old IL-10^{-/-} mice or 8-wk-old

MRL/Mp ($n = 6$ per group) mice i.p. with 5 mg/kg Poly IC (stock 1 mg/ml) three times a week. The duration of AIP induction in IL-10^{-/-} mice was 6 wk and in MRL/Mp mice 4 wk.

Treatment with LT β R-Ig

Twelve-mo-old Tg(Elal-LTa,b) and twelve-wk-old IL-10^{-/-} and MRL/Mp mice with established AIP were injected i.p. with 100 μ g LT β R-Ig fusion protein or with MOPC21 (control IgG) for 4 wk on a weekly basis. As MOPC21 treatment results did not differ significantly from untreated mice, data are not separately shown. After 4 wk of treatment, mice were sacrificed, pancreas was resected, and serum was obtained through cardiac puncture. LT β R-Ig fusion protein and with MOPC21 (control IgG) were provided by Biogen (Cambridge, MA).

Treatment with aCD4 and aCD20 Abs

Twelve-mo-old Tg(Elal-LTa,b) and twelve-wk-old IL-10^{-/-} and MRL/Mp mice with established AIP were injected i.p. with 200 μ g aCD20 or 200 μ g aCD4 Abs for 4 wk on a weekly basis. After 4 wk of treatment, mice were sacrificed, pancreas was resected, and serum was obtained through cardiac puncture. aCD20 Ab (clone 18B12-mIgG1) was provided by Biogen.

Serum antilactoferrin Ab test

Microtiter plates (MaxiSorp; Nalge NUNC, Roskilde, Denmark) were coated with 20 mg/ml of lactoferrin (LF) (Sigma Chemicals) in 100 ml of 50-mM sodium carbonate buffer (pH 9.5) and kept overnight at 4°C. The coated wells were washed three times with PBS and blocked with 5% nonfat milk in PBS for 2 h at 37°C. After three washes with PBS, each well was incubated with 100 ml each of mouse serum sample diluted at 1:10 with PBS containing 2% nonfat milk for 2 h at room temperature. The wells were then washed with PBS and incubated with phosphatase-conjugated goat anti-mouse Abs (BioSource) for 1 h at room temperature. After three washes with PBS, the bound Abs were detected with phosphatase substrate (Dako). The plates were read at 405 nm using an ELISA plate reader (Vmax; Molecular Devices, Tokyo, Japan).

IgG ELISA

The mouse IgG1 was quantified by Ready-SET-Go! ELISA (88-50410; eBioscience), according to the manufacturer's instructions. Serums from Tg(Elal-LTa,b), IL-10^{-/-}, and MRL/MP mice with AIP were used for the assay.

Flow cytometry

The freshly harvested pancreas was cut into pieces and digested in McCoy 5A Medium containing 10% FBS, 1 mg/ml collagenase D (11088858001; Sigma-Aldrich), and DNase I (10104159001; Sigma-Aldrich) on a shaker at 450 rpm for 25 min at 37°C. Cells were filtered with a cell strainer (100 μ M) followed by a centrifugation for 5 min at 1500 rpm, and the pellet was washed in PBS. Samples were incubated with Abs for at least 30 min in the dark at 4°C before washing in PBS and fixed in FACS buffer (50% PBS and 50 of 4% PFA). Isolated pancreatic immune cells were stained with

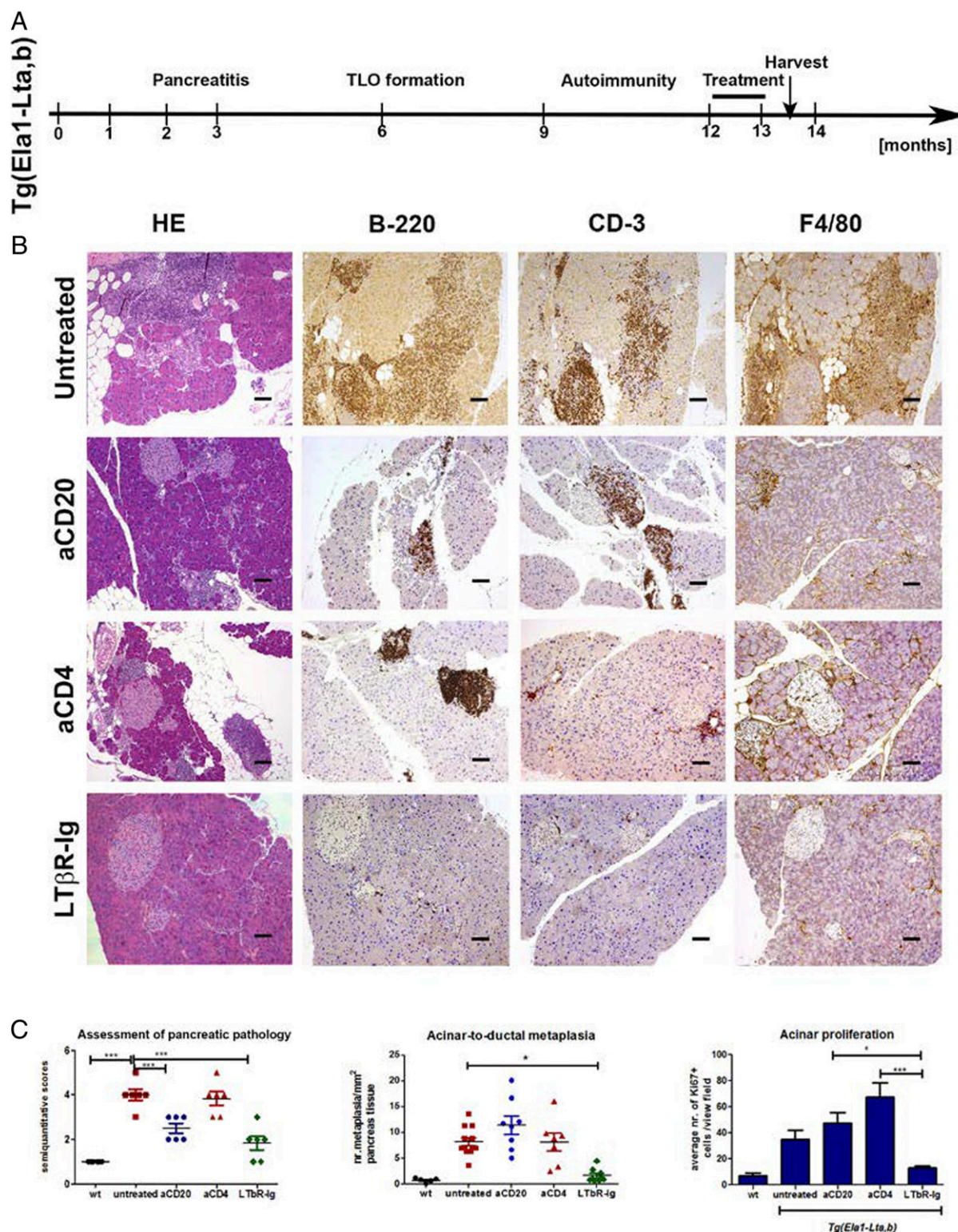


FIGURE 1. Treatment schedule and pancreatic histology.

(A) Schematic representation of AIP development and treatment schedule in Tg(Ela1-Lta,b) mice. (B) Immunohistochemical analysis of B cells (B220), T cells (CD3), and macrophages (F4/80) in 12-mo-old Tg(Ela1-Lta,b) mice upon treatments ($n = 10$). Stainings are representative of at least five independent experiments. (C) Pancreatic damage was assessed by a blinded observer after treatments ($n = 6$). Number of ADM quantified on H&E staining and normalized to the total surface of pancreas in Tg(Ela1-Lta,b) mice. Quantification of acinar cell proliferation based on counting of Ki67⁺ nuclei ($n = 8$). Scale bar, 100 μ m. * $p < 0.05$, *** $p < 0.001$.

CD45 (109826), CD8a (100711), CD4 (100453), CD19 (557399), F4/80 (123113), CD11c (117309), PCDA-1 (127009), CD49b (103503), CD45R/B220 (103221), and CD3ε (100305). All Abs mentioned above were purchased from BioLegend.

Samples were acquired using BD FACSCanto II (BD Biosciences) and analyzed with FlowJo v9.8.5 software (Tree Star).

Statistical analyses and software

GraphPad Prism version 5 (La Jolla, CA) was used to construct figures and diagrams. The data are presented as the mean \pm SEM. One-way ANOVA with Dunnett correction or unpaired *t* tests and Mann–Whitney tests were used where appropriate. Differences were considered statistically significant if $p < 0.05$ and were marked with an asterisk.

RESULTS

LTβR-Ig and aCD20 treatment improve AIP

Tg(Elal-Lta,b) mice spontaneously develop AIP at 9–12 mo of age. The inflammatory reaction in mice with AIP has been characterized by immunohistochemistry and flow cytometry (Fig. 1B, Supplemental Fig. 1D). The analysis showed a high accumulation of T and B cells as well as macrophages and a mild infiltration of plasmacytoid dendritic cells (pDCs) in mice with AIP. To compare the treatment efficacy of LTβR-Ig with depletion of B and CD4 T cells, mice with established AIP were injected with the respective treatment regimens starting 12 mo of age (Fig. 1A). During the treatments, the percentage of peripheral T and B cells were monitored in all three treatment groups (Supplemental Fig. 1A). As expected, injection with aCD20 Ab reduced peripheral B cells. Similarly, upon aCD4 Ab injection, a gradual decrease of CD4⁺ T cells was observed. However, in the LTβR-Ig treatment group, CD4⁺ T cell and B cell levels did not change.

We next assessed pancreatic pathology with a focus on the presence of inflammatory cells following treatments in the three treatment groups (Fig. 1B). As reported before (5), 12-mo-old untreated Tg(Elal-Lta,b) mice harbor large follicles constituting of T and B cells and an extensive number of infiltrating macrophages. LTβR-Ig treatment resulted in a significant decrease in B and T cell as well as macrophage infiltration. Periodic injection of aCD20 improved overall pancreatic pathology, mostly shown by more intact lobes and less infiltrating F4/80⁺ macrophages. Upon CD4⁺ T cell depletion, numerous lymphoid follicles, predominately filled with B cells with diffusely infiltrating T cells, remained in the pancreatic lobes. Macrophage influx in the aCD4 treatment group was higher than aCD20 treatment group. Blinded analysis of H&E staining showed the most prominent improvement in pancreatic histology in the LTβR-Ig treatment group and significant amelioration in the aCD20 group (Fig. 1C).

Blocking LTβR signaling prevented pancreatic injury

In response to injury, the pancreas activates regenerative processes to maintain tissue homeostasis. The prevailing concept is that after injury, 1) exocrine pancreatic cells (acinar, centroacinar, and

ductal cells) start to proliferate or 2) acinar cells dedifferentiate into ductal or progenitor cells as part of a self-defense mechanism or regeneration (17) (ADM). Therefore, we assessed the proliferation of acinar and inflammatory cells in the pancreas after the treatment regimens. Our results indicate that compared with the T and B cell depletion treatment, LTβR-Ig significantly reduced acinar cell proliferation (Fig. 1C); however, none of the treatments affected the proliferation of infiltrating cells (Supplemental Fig. 1B). As a regenerative response, we quantified acinar transdifferentiation by counting ADM. Only LTβR-Ig treatment significantly reduced ADM formation, ductal complexes, and acinar proliferation (Fig. 1C), as shown by the assessment of morphology on H&E (Supplemental Fig. 1C), transdifferentiation by amylase and CK19 staining, and Sox9 gene expression (Supplemental Fig. 1E, 1F). The other two treatments did not influence the acinar transdifferentiation. These results suggest that inhibiting LTβR signaling reduces injury signals, leading to diminished injury and hence less need for a regenerative response.

Inhibition of LTβR signaling moderates local inflammatory reaction

Acinar cell injury also leads to local inflammatory reaction. There is evidence showing that acinar cells can produce inflammatory mediators such as chemokines and cytokines (18). Additionally, infiltrating immune cells also contribute to the inflammatory signature. For that reason, after the three treatment regimens, we analyzed the above-mentioned factors (Fig. 2). LTβR-Ig and aCD20 treatment significantly reduced the inflammatory cytokine (*IL-6*, *IL-1β*, and *Mip3β*) and chemokine response (*Mcp1*), genes involved in lymphocyte activation and attraction (*Baff*, *Cxcl13*, and *Ccl20*), and further members of the TNF superfamily (*Tnfa* and *Light*). However, depletion of CD4⁺ T cells could only significantly affect *Ccl20* and *Cxcl13* gene expression. The adhesion molecule (*Vcam*) and expression of LT (*Ltα* and *Ltβ*) were not significantly affected by any treatment (Fig. 2).

We further evaluated the Th cell subsets on the mRNA level, based on their distinct cytokine expression profile and prototypic transcription factors (TF). During AIP, predominantly Th1 and Th2 type cells are present (5) in untreated mice (Fig. 3A). aCD20 treatment did not significantly influence the Th cell subsets, whereas aCD4 Ab significantly decreased Th1 and mainly Th2 response. On the contrary, LTβR-Ig treatment resulted in a significant reduction of T-bet and Th1 cytokines (Fig. 3A). No difference in the expression of Th17 cytokines was observed, supporting the hypothesis that Th17 cells are not involved in type 1 AIP pathogenesis (19). Although the TF Rorγt is upregulated in Tg(Elal-Lta,b) mice, this TF is also expressed by lymphoid tissue inducer cells and lymphoid tissue inducer-like cells (20). Tregs have also been shown to be elevated during AIP in humans and mice (5, 21, 22). All treatments were able to decrease the number of infiltrating Tregs (Fig. 3A). Treg-specific cytokines (*IL-10* and *TGF-β*) displayed a trend of lower expression in the LTβR-Ig and aCD20 treatment groups.

As rodents have no subclass of IgG4, we assessed IgG1 levels as it is suggested to serve as its counterpart in mice. We observed a

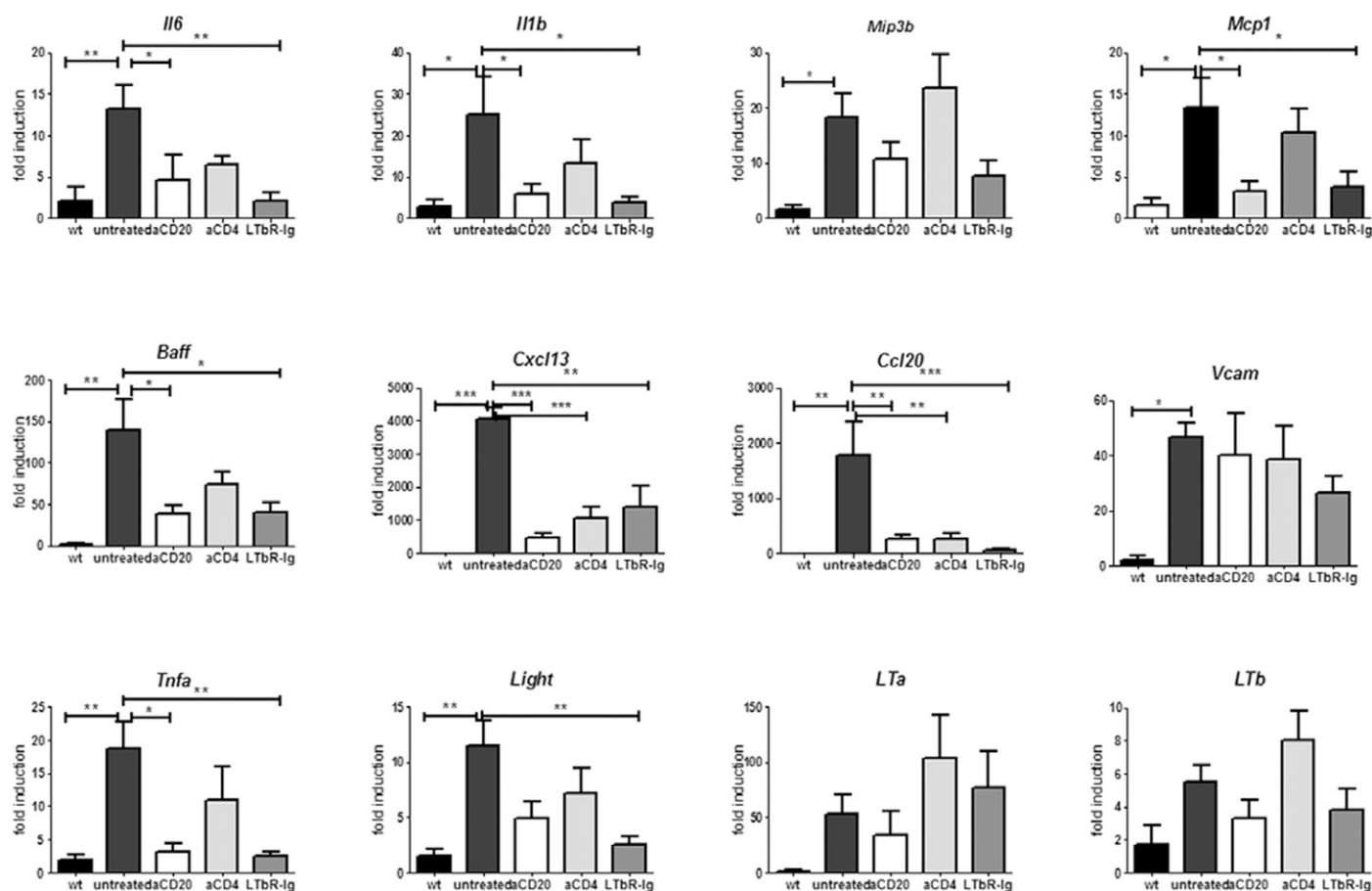


FIGURE 2. Expression analysis of genes involved in AIP.

Quantitative RT-PCR analysis of pancreatic tissue in Tg(Elai-Lta,b)-treated mice ($n = 5$) and in wild type littermates ($n = 4$). Experiments were performed in duplicates. Data are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

reduction in IgG1 as a result of LT β R-Ig treatment but not upon aCD20 (Supplemental Fig. 4D).

To define the cellular and molecular mechanisms of the treatment response, we analyzed the activation of two key TFs involved in pathological processes in the pancreas, including AIP (5): NF- κ B and Stat3 (23, 24) (Fig. 3B, Supplemental Fig. 2). In untreated Tg(Elai-Lta,b) mice, prominent Stat3 phosphorylation was detected on acinar and inflammatory cells. Quantification of Stat3-positive nuclei showed that aCD20 and LT β R-Ig treatments significantly reduced Stat3 activation in acinar cells but not in inflammatory cells (Fig. 3B, Supplemental Fig. 2B). aCD4 treatment did not show any beneficial effect on Stat3 activation. We further identified pronounced nuclear translocation of RelB in the acinar cells and inflammatory cells, in contrast to RelA, which remained mainly cytoplasmic (Supplemental Fig. 2A). Conversely, the activation of the noncanonical NF- κ B signaling was only influenced by LT β R-Ig treatment as blocking LT β R signaling significantly reduced RelB nuclear translocation on acinar and inflammatory cells. The results of the detailed molecular characterization of the treatments are summarized in Table I.

LT β R-Ig could sufficiently disrupt tertiary lymphoid organs

Tertiary lymphoid organs (TLOs) are important inductive sites for self-reactive T lymphocytes and Abs that contribute to pathology in autoimmune diseases. Only LT β R-Ig treatment could successfully disrupt characteristic structures of TLOs, like high endothelial venules (PNad), follicular dendritic cell networks (FDCM1), and germinal center B cells (CD21/35) (Fig. 4A). In addition, the proportion of CD4 $^{+}$ and CD8 $^{+}$ T cells in the follicles was reduced. We noticed significantly lower autoantibodies directed against LF in LT β R-Ig- and aCD20-treated groups (Fig. 4B).

IgG4-related disease is a systemic disease including involvement of other organs (e.g., salivary gland, bile ducts, or manifestations in the kidney) (5). Therefore, the incidence of IgG deposits in the mesangium and capillaries in the kidneys upon different treatment modalities were analyzed by light microscopy. Our results indicate that all three treatments could sufficiently reduce the extrapancreatic manifestation in the kidney (Fig. 4C). Assessments of bile ducts and salivary glands showed no morphological alterations in untreated (Tg(Elai-Lta,b) mice (Supplemental Fig. 2D and data not shown).

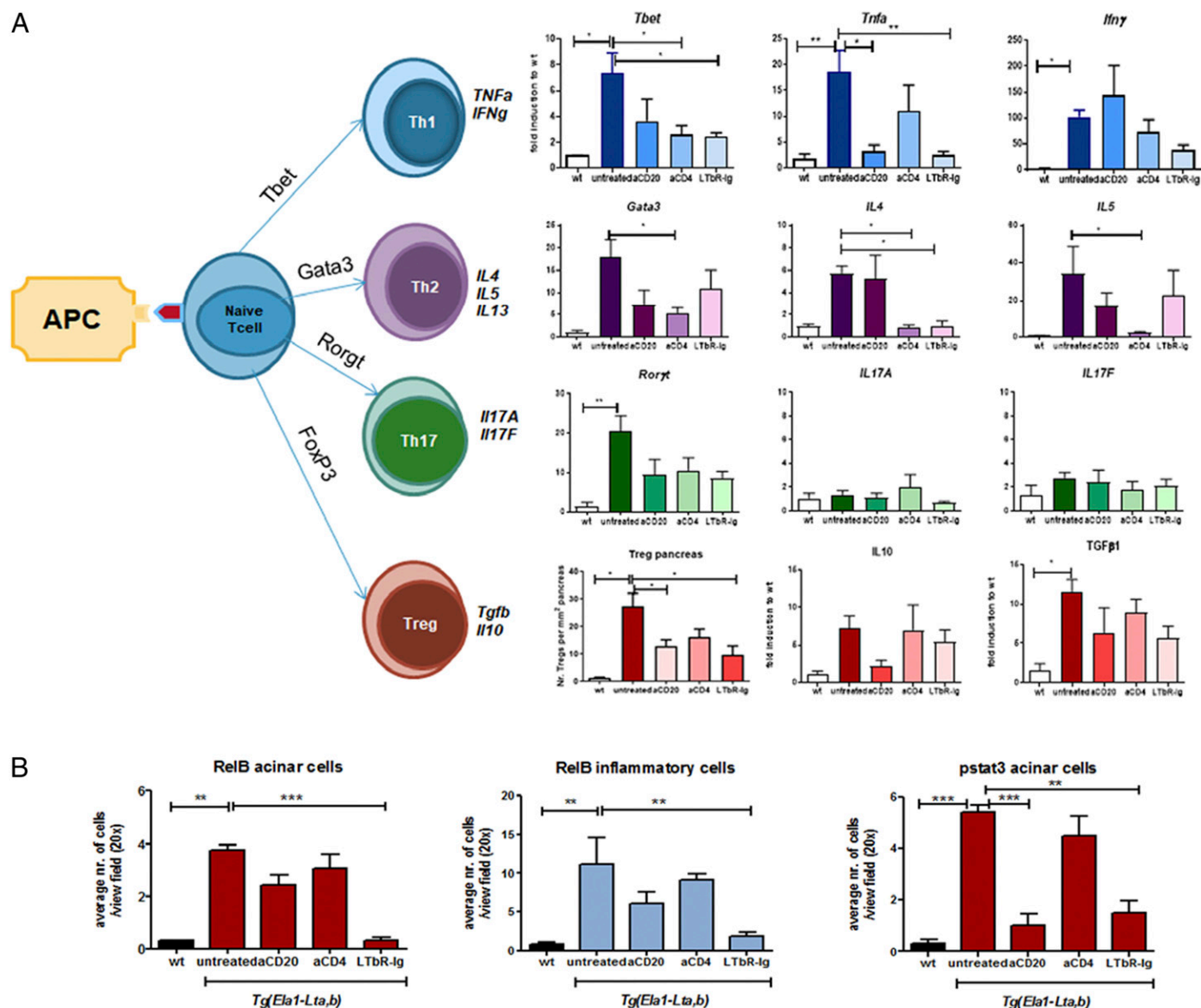


FIGURE 3. Cellular and molecular mechanisms of treatment response.

(A) Quantitative RT-PCR analysis of pancreatic *Tnf- α* , *Ilfn- γ* , *Tbet*, *Gata3*, *Il4*, *Il5*, *Ror γ t*, *Il17*, *Tgfb*, and *Il10* transcripts in untreated 12-mo-old *Tg(Elal-Lta,b)* mice and mice treated with aCD20, aCD4, and LT β R-Ig ($n = 5$). Experiments were performed in duplicates. Data are representative of two independent experiments. (B) Quantification of Stat3⁺ acinar cells and RelB nuclear translocation in acinar cells and in infiltrating immune cells of 12-mo-old *Tg(Elal-Lta,b)* ($n = 4$ untreated, $n = 5$ treated mice) and C57BL/6 controls ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

AIP development is associated with LT β R signaling in IL-10^{-/-} and MRL/Mp mouse models

To validate the effect of LT β R-Ig treatment and evaluate immune cell depletion in AIP, we studied two additional experimental mouse models of AIP. MRL/Mp (12) and IL-10^{-/-} mice (13) have been shown to develop type 1 AIP after repetitive injection with Poly IC. The induction and progression of AIP as well as the treatment allocation is depicted in Fig. 5A and 5B. Given that LT β R signaling plays an important role in AIP development in mice and humans (5), we first investigated the expression of LT β R ligands in

AIP of MRL/Mp and IL-10^{-/-} mice (Fig. 5C). The ligands Lt α , Lt β , and Light were significantly upregulated in both models, supporting that LT β R signaling contributes to the development of AIP. Furthermore, an activation of the noncanonical NF- κ b signaling was observed in the pancreas of MRL/Mp mice (Supplemental Fig. 2C) upon Poly IC injection, similar to the *Tg(Elal-Lta,b)* mice. This finding further verifies the involvement of LT α and β and the noncanonical NF- κ b signaling in AIP and makes the MRL/Mp and IL-10^{-/-} models applicable to study the effects of LT β R-Ig and immune cell depletion.

TABLE I. Summary of molecular characterization of the treatment groups

		Treatment groups		
		<i>LTβR-Ig</i>	<i>aCD20</i>	<i>aCD4</i>
Autoantibody production		↓	↓	■
Acinar cell proliferation and ADM formation		↓	■	■
Extrapancreatic manifestation		↓	↓	↓
TLO integrity		↓	■	■
Activation of transcription factors		Stat3 ↓ NF-κB ↓	Stat3 ↓ NF-κB ■	Stat3 ■ NF-κB ■
Cytokine and chemokine expression	Inflammatory cytokines (Il-6, Il1b, Mip3b)	↓	↓	■
	Inflammatory chemokines (Mcp1)	↓	↓	■
	Lymphocyte activation and attraction (Baff, Cxcl13, Ccl20)	↓	↓	■
	TNF superfamily (TNFa, Light)	↓	↓	■
	Adhesion molecule (Vcam)	■	■	■
Inflammatory cell distribution	T helper cell subsets	Th1 ↓ Th2 ■ Th17 ■	Th1 ■ Th2 ■ Th17 ■	Th1 ↓ Th2 ↓ Th17 ■
	Regulatory T-cells	↓	↓	■
	Inflammatory cell infiltration	↓	↓	■

unchanged: ■ reduced: ↓

Validating the therapeutic efficacy of *LTβR-Ig* and *aCD20* in *LT-independent* mouse models

In MRL/Mp and IL-10^{-/-} mice, the treatments started when mice already had established autoimmunity. Mice were subjected to weekly i.p. injections of the respective therapy for 4 wk and harvested consecutively (Fig. 5B). Treatment efficacy was assessed by 1) histological evaluation of infiltrating inflammatory cells and TLOs (Fig. 6), 2) cytokine and chemokine expression (Supplemental Figs. 3A, 4A), and 3) quantification of autoantibodies against pancreatic Ags (LF) (Supplemental Figs. 3B, 4B).

In the MRL/Mp model, severe histopathological changes were observed after Poly IC administration, replacing almost all pancreatic lobes (marked inflammatory cell infiltration with complete destruction of acini and pancreatic parenchyma, ductal complexes, and strong fibrosis) (Fig. 6A). *LTβR-Ig* and *aCD20* treatment did not result in a significant benefit in the MRL/Mp model, based on the histological evaluation. However, *aCD20* injection showed a trend of reduced inflammatory gene expression (Fig. 6C, Supplemental Fig. 3A) and reduced autoantibody level (Supplemental Fig. 3B) but no

changes in Igs (Supplemental Fig. 4D). In agreement with the previous experiment in Tg(Elal-Lta,b) mice, *LTβR-Ig* but not *aCD20* could significantly reduce the number of TLOs in the pancreatic parenchyma of MRL/Mp mice; however, numerous TLOs still remained in the tissue (Fig. 6C).

In IL-10^{-/-} mice, administration of Poly IC induced a more consistent, specific destruction of the exocrine pancreatic tissue but milder than observed in the MRL/Mp mice (Fig. 6B). Upon both *LTβR-Ig* and *aCD20* treatment, IL-10^{-/-} AIP model had significantly reduced diffuse inflammatory infiltration, ADM, and edema almost similar to Tg(Elal-Lta,b) mice. Furthermore, pancreatic cytokine (*Il6* and *Il1b*) and chemokine (*Mcp1*) expression and genes involved in lymphocyte activation and attraction (*Baff*, *Cxcl13*, and *Ccl20*) were also decreased (Supplemental Fig. 4A). Measurement of pancreatic autoantibodies and IgG1 also revealed a strong trend of reduction with both treatments (Supplemental Fig. 4B, 4D). Next, we assessed acinar cell proliferation based on Ki67⁺ by immunohistochemistry. Our results indicate, similarly to Tg(Elal-Lta,b) mice, that only *LTβR-Ig* treatment was able to significantly reduce acinar cell proliferation in mice with AIP (Supplemental Fig. 4C).

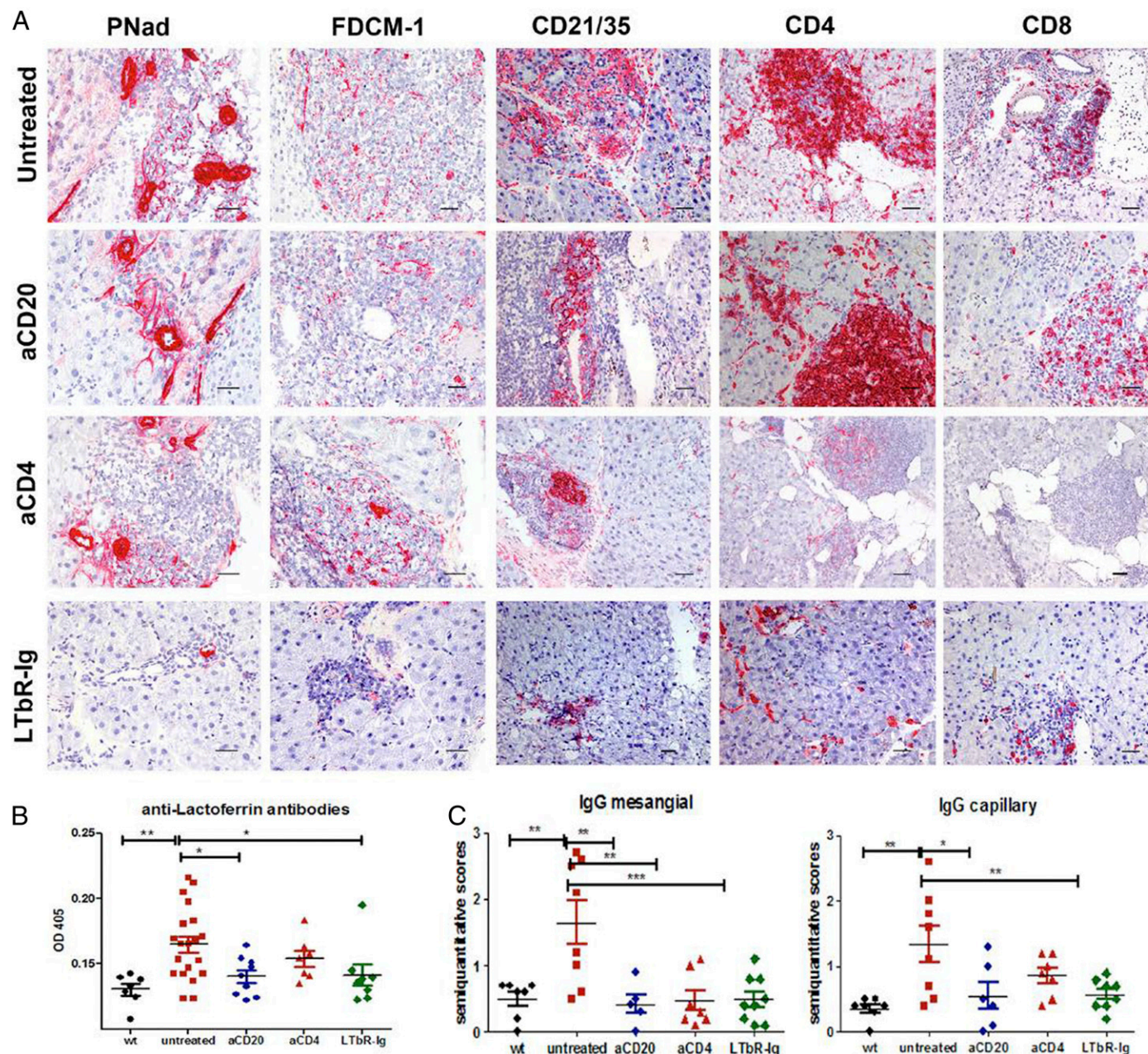


FIGURE 4. Characterization of autoimmunity.

(A) Characterization of TLOs by visualizing PNAd⁺ high endothelial venules, FDCM-1⁺ follicular dendritic cell networks, CD21/35⁺ germinal center B cells, CD4⁺ T cells, and CD8⁺ cytotoxic T cells in a follicle in 12-mo-old mice (scale bar, 50 μ m). Stainings are representative of at least three independent experiments. (B) Autoantibodies against LF were measured from the serum of transgenic mice by ELISA ($n = 6$ wild type; $n = 20$ untreated, and $n = 8$ treated groups). Experiments were performed in duplicates and repeated three times. (C) Assessment of extrapancreatic manifestations in the kidneys of 12-mo-old Tg(Ela1-LTab) mice ($n =$ minimum 7). The stainings for the experiment represent two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We thereby confirmed the efficacy of LT β R-Ig and aCD20 treatments to alleviate AIP induced in IL-10^{-/-} mice.

DISCUSSION

AIP is a recently discovered disease of the pancreas, with suspected autoimmune causes. To understand AIP, we previously

developed a novel transgenic mouse model mimicking human AIP. The model is based on the overexpression of LT in the pancreas, which results in the development of type I AIP, with a robust penetrance and phenotypic reproducibility. In this study, we used this novel genetic model of AIP to analyze the detailed molecular mechanisms of LT β R-Ig treatment to alleviate AIP. The strength of the study is that we validated the efficacy of LT β R-Ig treatment

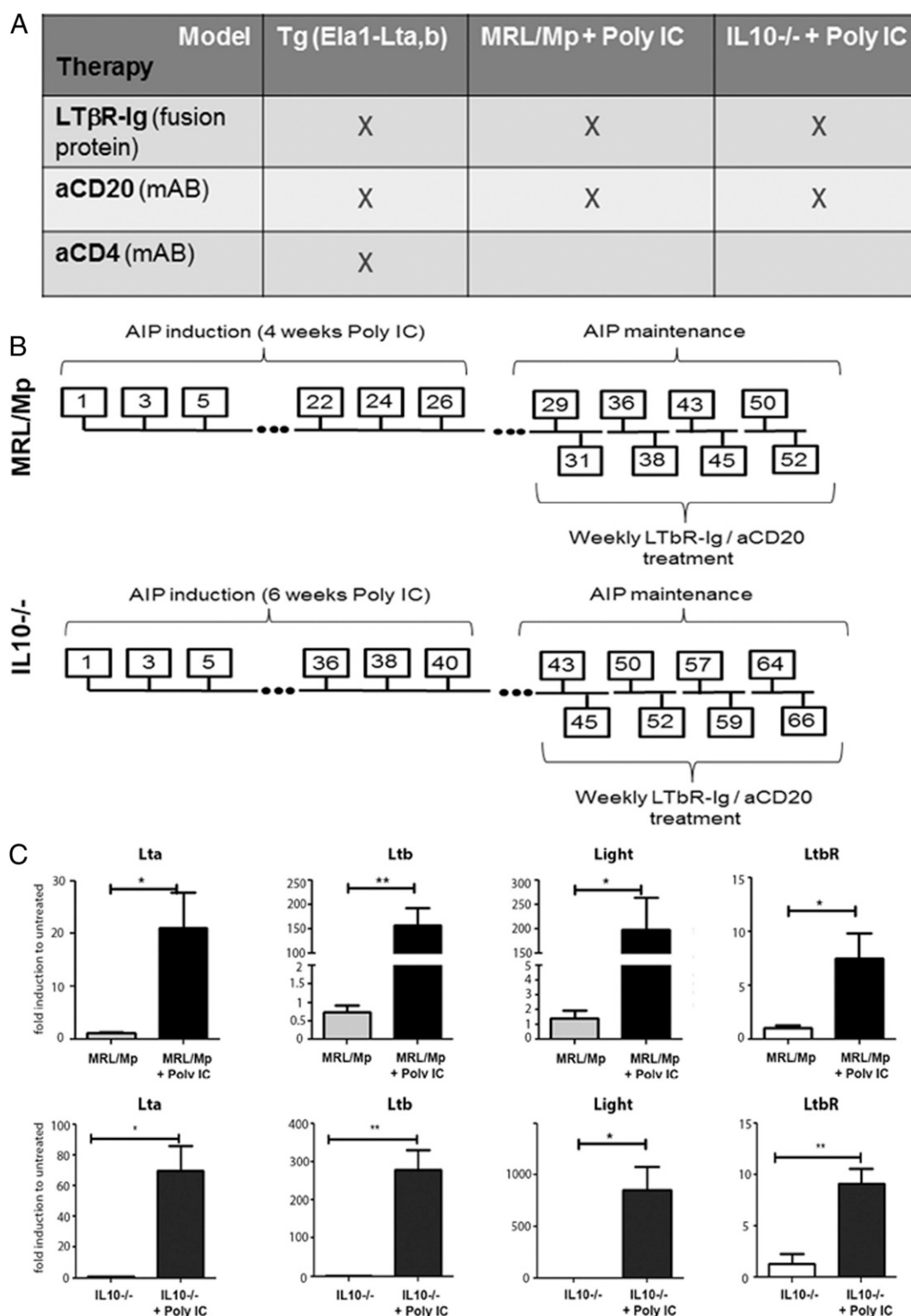


FIGURE 5. Treatment schedule and characterization of MRL/Mp and IL10^{-/-} models of AIP.

(A) Table showing the experimental setup, summarizing which treatment was performed in which mouse model. (B) Treatment schedule for treating AIP in MRL/Mp and IL10^{-/-} mice. Numbers in boxes represent experimental days. (C) Quantitative RT-PCR analysis of Lta, Ltb, Light, and LtβR genes. Transcripts were analyzed in mRNA from pancreatic homogenates of MRL/Mp and IL10^{-/-} mice with established AIP (n = 7). Experiments were performed in duplicates. Data are representative of two independent experiments. *p < 0.05, **p < 0.01.

in another animal model of AIP (IL-10^{-/-} induced with Poly IC), which develops AIP without transgenic overexpression of LT. Besides investigating the effector mechanisms of LTβR-Ig, we also tested therapeutic strategies that seems to be beneficial for AIP patients (i.e., depletion of CD20⁺ B cells and CD4⁺ T cells).

The principle finding of the study is that LTβR-Ig and aCD20 treatment were efficient to alleviate AIP in two animal models of AIP [Tg(Elai-Lta,b) and IL-10^{-/-}]. Both treatments led to a significant decrease in autoantibody production and inflammatory cell infiltration in the pancreas. The molecular mechanisms of this beneficial effect possibly involve the downregulation of Stat3 and noncanonical NF-κB activation. Importantly, LTβR-Ig proved to be more efficient in reducing the TLO burden, which makes it a valuable candidate for the treatment of AIP. aCD4 treatment resulted in reduced Th1 and Th2 polarization; however, this did not alleviate AIP.

LTβR-Ig not only more efficiently reduced TLOs, but in contrast to aCD20 and aCD4 treatments, it could significantly decrease the proliferation rate of acinar cells and reduce the number of ADMs. A higher proliferation rate in the aCD20 group

can also imply an increased regenerative process. However, in the LTβR-Ig-treated group, the lower proliferation is proportional to less ADM, which indicates that regeneration already took place as ADMs are reversible. Our findings of the phenotypic characterization of Tg(Elai-Lta,b) mice upon the different treatment regimens are summarized in Table I. This table clearly shows that LTβR-Ig treatment is associated with the most beneficial effects on AIP pathogenesis (Table I).

These findings highlight an important point concerning the immunopathogenesis of AIP. In recent years, two main theories were proposed. The first concept involves an abnormal adaptive immune response, leading to an increased accumulation of mostly Th2 cells. Their cytokine production (e.g., IL-4, IL-10, and IL-13) can promote IgG production by B cells, contributing to the pathogenesis of AIP (25).

According to the second theory, T cells are secondary, and innate immunity plays a fundamental role in AIP development. Initial evidence for that was provided from studies showing that B cells can induce IgG4 production by APCs (26, 27). Monocytes,

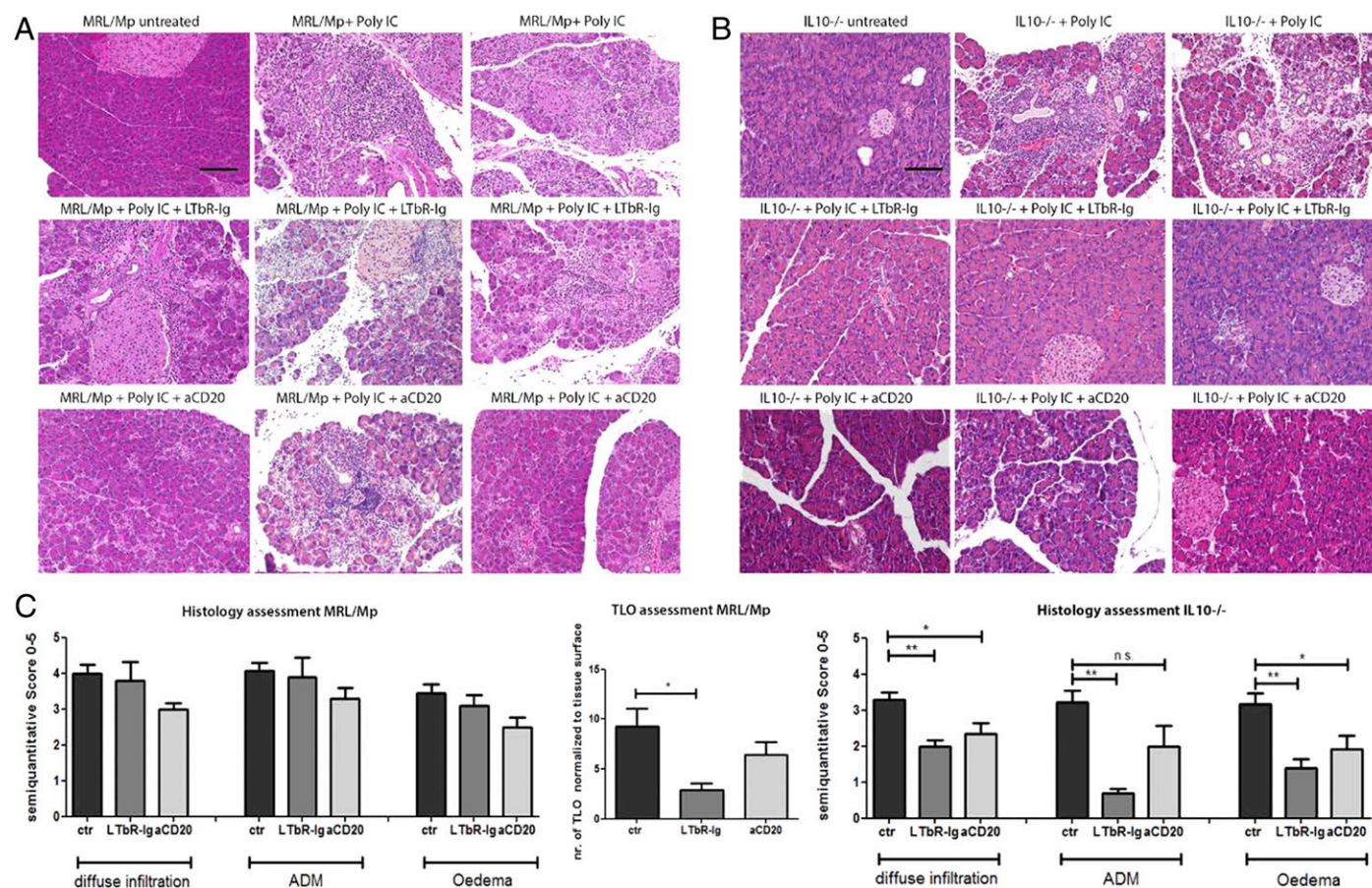


FIGURE 6. Histological assessment of treatments in MRL/Mp and IL-10^{-/-} models of AIP.

(A) Pancreatic morphology visualized on H&E staining in one untreated MRL/Mp mice, two mice with AIP (treated with Poly IC), and three mice with AIP and treated with LTβR-Ig or aCD20. Scale bar, 100 μm. (B) Pancreatic morphology visualized on H&E staining in an untreated IL-10^{-/-} mice, two mice with AIP (treated with Poly IC), and three mice with AIP and treated with LTβR-Ig or aCD20. Scale bar, 100 μm. (C) Histological assessment of a blinded observer in the MRL/Mp and IL-10^{-/-} model of AIP. Mice treated with Poly IC only (control $n = 8$) were compared with mice treated with LTβR-Ig ($n = 5$) or aCD20 ($n = 5$). Stainings are representative of two to three independent experiments. * $p < 0.05$, ** $p < 0.01$.

basophils, and importantly, pDCs secreting IFN- α and BAFF can induce IgG secretion by B cells in a T cell-independent manner. Further evidence has been provided that pDCs are not only associated with the development of murine AIP (MRL/Mp) but also chronic fibroinflammatory responses are mediated by activated pDCs producing IFN α and IL-33 (26).

Our results support the second concept as aCD4 treatment significantly reduced Th2 cytokines but did not achieve any benefits in terms of AIP pathology. Furthermore, in Tg(Elal-Lta,b) mice, we detected slightly increased numbers of pDCs, and LT β R-Ig and aCD20 treatments significantly reduced BAFF expression. Our results do not identify the cellular source of BAFF. Because pDCs produce high levels of IFN- α in response to a stimulus, it is possible to observe sudden IFN- α in MRL/Mp after stimulus (Poly IC). However, in the genetic Tg(Elal-Lta,b) model, lack of an external acute stimulus targeting pDC or other cells explains undetectable IFN- α levels in the serum.

Interestingly, we measured high LT expression in MRL/Mp model, but the administration of LT β R-Ig did not improve AIP in terms of pancreatic morphology and inflammatory gene expression. The method of AIP initiation is stimulation of TLR3 through a synthetic dsRNA (Poly IC), which is conceptually different from the induction of the LT β R and TNF pathways through pancreatic LT overexpression. We would nonetheless like to point out that LT β R-Ig treatment led to reduced tertiary lymphoid tissues in the MRL/Mp model, which is an important pathological feature of AIP. However, AIP induced by Poly IC in the IL-10 $^{-/-}$ mice responded well to LT β R-Ig treatment, ruling out an induction-specific cause. It may be noted that MRL/Mp mice, which are not on C57BL/6 background, develop a systemic autoimmunity that may hinder a response to LT β -Ig treatment, whereas the genetic background of IL-10 $^{-/-}$ mice is C57BL/6, identical to Tg(Elal-Lta,b) mice. MRL/Mp mice exhibit massive destruction of pancreatic architecture, and a potential regeneration could not be supported by blocking LT β R signaling or depleting B cells (aCD20). Therefore, further clinical studies with LT β R-Ig in human AIP should focus on early/newly diagnosed disease or used as a second-line treatment after the amelioration of inflammation by steroid treatment. Plausibly, genetic predisposition of human AIP patients (28, 29) might influence the treatment success, which should be investigated in upcoming clinical studies.

In conclusion, in this comparative study, we provide mechanistic support for two treatments that can be used to treat AIP patients. We suggest introducing new therapeutic interventions (i.e., LT β R-Ig and a treatment with rituximab [aCD20] that was already tried on a small cohort of AIP patients).

DISCLOSURES

The authors have no financial conflicts of interest.

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